AN INVESTIGATION INTO THE USE OF CARDIOLIPIN ANTIGENS*

II. COMPLEMENT-FIXING PROPERTIES OF CARDIOLIPIN WASSERMANN ANTIGEN

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The mode of approach adopted whilst investigating the complement-fixing properties of cardiolipin Wassermann antigens was similar to that used in the case of their anticomplementary characteristics, namely, the various constituents of the antigen were examined individually, severally, and collectively.

Complement-Fixing Properties of Cardiolipin

This reagent was examined using two ranges of 0·6 per cent. to 0·0045 per cent. One range was suspended in saline at a dilution of 1 in 50 and the other at 1 in 200. No fixation of complement was obtained when using syphilitic sera.

Mixtures of cardiolipin and lecithin were next tested for their complement-fixing ability in the presence of a suitable serum. The results (Table VII) show that, with a constant lecithin content of 1·7 per cent., fixation of complement occurs when the antigens contain from 0·05 per cent. to 0·15 per cent. cardiolipin in saline suspensions of 1 in 200.

TABLE VII

COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNT OF CARDIOLIPIN IN CARDIOLIPIN-LECITHIN ANTIGENS

Antigens†		Approximate Ratios of Cardiolipin to Lecithin	Quantitative Wassermann Reaction Results with Syphilitic Serum (P.P.R.* titre positive 1 in 16)
Cardiolipin (Percent.)	Lecithin (Percent.)		
0·15	1·7	1–10	Positive serum diluted 1–8
0·075	1·7	1–20	Positive serum diluted 1–2
0·05	1·7	1–34	Positive with neat serum
0·025	1·7	1–68	Negative
0·0125	1·7	1–136	Negative
0·15	0·17	1–1	Negative
0·075	0·17	1–2	Negative
0·05	0·17	1–3·4	Negative
0·025	0·17	1–6·8	Negative
0·0125	0·17	1–13·6	Negative

* P.P.R. = Price's precipitation reaction.
† Made in saline suspensions of 1/200.

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As the strength of the cardiolipin increases, so does the sensitivity of the antigen, as evidenced by the rise in the titre of the serum. With a series of cardiolipin-lecithin antigens, each series containing 0·17 per cent. lecithin, no fixation occurs.

Next, antigens made up of mixtures of cardiolipin and cholesterol were tested. Table VIII shows that within the limits of the experiment a mixture of these two reagents used as antigens failed to fix complement.

TABLE VIII

COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNTS OF CARDIOLIPIN IN CARDIOLIPIN-CHOLESTEROL MIXTURES

Antigens*		Fixation of Complement in Presence of positively reacting Syphilitic Serum
Cardiolipin (Percent.)	Cholesterol (Percent.)	
0·15 decreasing to 0·0125	0·5	Nil
	0·5	Nil
0·15 decreasing to 0·0125	0·25	Nil
	0·25	Nil

* Made in saline suspension of 1/200

Finally, to complete this part of the investigation, mixtures containing the three components of cardiolipin Wassermann antigen were prepared. Five antigens were made, each containing lecithin 0·05 per cent. and cholesterol 0·5 per cent. but using *increasing* doses of cardiolipin from 0·015 per cent. to 0·15 per cent. Two other antigens were made, each containing lecithin 0·005 per cent. and cholesterol 0·5 per cent, one with 0·0075 per cent. and the other with 0·0037 per cent. cardiolipin. The optimal titres and the anticomplementary titres of the seven antigens were determined by means of optimal proportions. The composition is shown in Table IX (opposite).

Twenty-four sera were now tested quantitatively in parallel using the seven antigens.

TABLE IX

KEY TO ANTIGENS USED TO DEMONSTRATE THE COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNTS OF CARDIOLIPIN IN CARDIOLIPIN WASSERMANN REACTION ANTIGENS

Antigen	Percent. (and Ratio) of Contents			Optimum Titre	Anticomplementary Titre in Absence of Normal Serum
	Cardiolipin	Lecithin	Cholesterol		
1	0·15 (1)	0·05 (0·33)	0·5 (3·33)	1/320	1/160
2	0·075 (1)	0·05 (0·66)	0·5 (6·6)	1/480	1/80
3	0·05 (1)	0·05 (1)	0·5 (10)	1/480	Nil
4	0·025 (1)	0·05 (2)	0·5 (20)	1/180	Nil
5	0·015 (1)	0·05 (3)	0·5 (33)	1/100	Nil
6	0·0075 (1)	0·005 (0·66)	0·5 (66)	1/60	Nil
7	0·0037 (1)	0·005 (1·3)	0·5 (135)	1/60	Nil

Table X shows that the most sensitive antigens are 2, 3, and 4; and that 3 (cardiolipin 0·05 per cent., lecithin 0·05 per cent., cholesterol 0·5 per cent.) is slightly better than the other two.

The conclusions to be drawn from this work may be summarized as follows:

(1) Cardiolipin and mixtures of cardiolipin and cholesterol are incapable of fixing complement in the presence of reagin.

(2) (*a*) A mixture of cardiolipin and lecithin, if suitably adjusted, can fix complement in the presence of syphilitic reagin. It should be emphasized, however, that it has been found that this fixation is at a very much lower level than that which occurs with same sera using cardiolipin antigens containing the three components at their optimal proportions. Fixation occurred when the lecithin was kept constant at 1·7 per cent. and cardiolipin varied from 0·05 per cent. to 0·15 per cent. The greater

TABLE X

COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNTS OF CARDIOLIPIN IN CARDIOLIPIN WASSERMANN REACTION ANTIGENS

Serum No.	Quantitative P.P.R. Results*	Cardiolipin Antigens with Serum Titre Recordings †						
		1	2	3	4	5	6	7
1	Negative	2	4	4	4	4	2	2
2	2	8	16	16	16	16–	8	8
3	1	2	8	8	8	4	4	2
4	8	8–	16–	16	16–	16–	16–	4
5	16	8	32–	32	32–	16	16	16–
6	1	Negative	Negative	4	1	1	1	Negative
7	32	16–	32	32	32	32	32–	16
8	32	8	16	32–	16	16	16	16
9	Negative	4–	4	8	8	8	4	2
10	32	4	8	16–	8	8	8–	4
11	2	2	4–	8–	8–	4	4	4–
12	2	2–	2	2	2	2–	2–	Negative
13	8	2–	4	4	4	4	4	2–
14	4	1	4–	4	4	4	4	1
15	8	16–	16	16	16–	16–	16–	8
16	1	Negative	Negative	Negative	Negative	Negative	Negative	Negative
17	2	4	8	8	8	8	8	4
18	1	4	8	8	8	8	8	4
19	2	4	8	8	8	8	8	4
20	4	2	4	8	4	4	4	4
21	8	4	4	4	4	2	2	1
22	4	4–	8	8	8	8	8	4
23	Negative	1	2	4–	2	2	2	2
24	8	4	16–	16–	16–	8	8–	4

* Expressed as Negative; 1 = positive with neat serum; or Numeral = positive with serum diluted 1 in ——

† { Numerals = full fixation serum diluted 1 in ——
{ Numerals – = 50 per cent. haemolysis serum diluted 1 in ——
{ Negative = 100 per cent. haemolysis.

the amount of cardiolipin, the greater the fixation (see Table VII, p. 78).

(*b*) Lecithin must be present in sufficient strength if fixation of complement is to occur in the presence of syphilitic reagin (see Table VII, p. 78).

It follows that if lecithin is used at a sufficient constant strength the amount of reagin demonstrated in a serum depends on the amount of cardiolipin present.

(3) The presence of cholesterol in an antigen which contains cardiolipin and lecithin results in a marked increase in the ability of these antigens to fix complement in the presence of syphilitic reagin (compare W.R. titres with PPR titres using same serum in Table VII, p. 78 and in Table X, p. 79).

(4) Using mixtures of the three reagents as antigens, if the lecithin and cholesterol are kept constant (at 0·05 per cent. and 0·5 per cent. respectively), the most sensitive antigen is obtained when the formula used is cardiolipin 1, lecithin 1, cholesterol 10.

Complement-Fixing Properties of Lecithin

The examination of lecithin was now undertaken, using two ranges of from 0·05 per cent. to 3·4 per cent., each strength being made up in saline suspensions of 1 in 25 and 1 in 100 respectively. No complement fixation was obtained when syphilitic serum was used.

Following on this, mixtures of lecithin and cardiolipin (constant) were tested, made up in suspensions of 1 in 200 of saline.

Table XI shows that, using antigens of constant cardiolipin content of 0·15 per cent. plus lecithin 0·2 per cent. or more, reagin can be demonstrated if the antigens are made up in a saline suspension of 1 in 200. It is to be noted that, if the lecithin in the antigen is increased above 0·4 per cent., sensitivity is not increased (compare Table VII, p. 78).

TABLE XI

COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNTS OF LECITHIN IN LECITHIN-CARDIOLIPIN ANTIGENS

Antigens*		Approximate Ratio of Lecithin to Cardiolipin	Quantitative Wassermann Reaction Results with Syphilitic Serum (P.P.R. titre positive 1 in 32)
Lecithin (percent.)	Cardiolipin (percent.)		
1·7	0·15	10–1	Positive serum diluted 1 in 8
0·85	0·15	5–1	Positive serum diluted 1 in 8
0·4	0·15	2·5–1	Positive serum diluted 1 in 8
0·2	0·15	1·25–1	Positive serum diluted 1 in 4
0·1	0·15	0·6–1	Negative
0·05	0·15	0·3–1	Negative
0·025	0·15	0·15–1	Negative
Range as above	0·05	..	Negative

* Made in saline suspension of 1/200

Ranges of lecithin from 1·7–0·0125 per cent. were now mixed with (*a*) 1·0 per cent. cholesterol and (*b*) 0·5 per cent. cholesterol. No fixation was obtained in the presence of syphilitic serum.

The next step was to make antigens containing constant amounts of cardiolipin (0·05 per cent.) and cholesterol (0·5 per cent.) with increasing amounts of lecithin ranging from 0·005 per cent. to 0·5 per cent. This is shown in Table XII.

TABLE XII

KEY TO ANTIGENS USED TO DEMONSTRATE THE COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNTS OF LECITHIN IN CARDIOLIPIN WASSERMANN REACTION ANTIGENS

Antigen	Percent. (and Ratio) of Contents			Optimum Titre	Anticomplementary Titre in Absence o Normal Serum
	Lecithin	Cardiolipin	Cholesterol		
1	0·5 (10)	0·05 (1)	0·5 (10)	1/640	Nil
2	0·25 (5)	0·05 (1)	0·5 (10)	1/640	Nil
3	0·05 (1)	0·05 (1)	0·5 (10)	1/480	Nil
4	0·01 (0·2)	0·05 (1)	0·5 (10)	1/480	Nil
5	0·005 (0·1)	0·05 (1)	0·5 (10)	1/400	Nil

After estimation of their optimal proportion titres, these antigens were tested out with 24 syphilitic sera. Table XIII (opposite) shows that the most sensitive antigen is Number 3, *i.e.* that containing lecithin 0·05 per cent., cardiolipin 0·05 per cent., and cholesterol 0·5 per cent., with the formula lecithin 1, cardiolipin 1, cholesterol 10. It is interesting to compare Table XIII with Table X (p. 79), which does not show such a great variation in the serum titres obtained with the antigens containing different amounts of cardiolipin.

As a result of this section of the work, the following conclusions can be drawn.

(1) Lecithin by itself, and mixtures of lecithin and cholesterol, when used as antigens, fail to fix complement in the presence of syphilitic serum.

(2) Suitable mixtures of lecithin and cardiolipin, can fix complement in the presence of syphilitic serum, *e.g.*

(*a*) when used at a constant cardiolipin strength of 0·15 per cent. and 0·2 per cent. or more of lecithin.

It should be noted that if the amount of lecithin is increased above 0·4 per cent., the sensitivity of the antigen is not increased (see Table XI).

(*b*) when used at a constant lecithin strength of 1·7 per cent. and 0·05 per cent. or more of cardiolipin (see Table VII, p. 78).

(3) When cholesterol is added to lecithin and cardiolipin, there is a marked increase in the sensitivity.

(4) Under the conditions of these experiments, using antigens containing constant amounts of cardiolipin (0·05 per cent.) and cholesterol (0·5 per cent.) with increasing amounts of lecithin (0·005 per cent. to 0·5), the most sensitive antigen is that based on the formula lecithin 1, cardiolipin 1, cholesterol 10 the unit strength of which is 0·05 per cent.

TABLE XIII

COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNTS OF LECITHIN IN CARDIOLIPIN WASSERMANN REACTION ANTIGENS

Serum No.	Quantitative P.P.R. Results	Cardiolipin Antigens with Serum Titre Recordings				
		1	2	3	4	5
1	4	8	8	16	16	Negative
2	1	32	32	64	32	Negative
3	4	Negative	Negative	32	Negative	Negative
4	2	8	16	32	16	Negative
5	4	32	32	64	32	4–
6	2	16	16	32	16	Negative
7	4	16	16	32	32	Negative
8	2	32	32	64	32	Negative
9	2	16–	16–	32	16	4–
10	1	Negative	Negative	8	Negative	Negative
11	Negative	Negative	Negative	16–	16–	8–
12	2	16–	16	64–	32	16
13	2	Negative	Negative	8	8–	Negative
14	2	8–	8–	64–	16–	Negative
15	8	16	32–	64	64	32–
16	4	8	16–	32	32–	Negative
17	64	16–	16	64–	64–	32–
18	16	32–	32–	64	64–	32
19	32	8–	16	32	16	16
20	128	64	128	256	256–	128
21	64	64	64	128	128	128–
22	2	16–	32–	32	64–	64–
23	2	4–	8–	16–	16–	16–
24	2	Negative	4–	16	32–	32–

Complement-Fixation Properties of Cholesterol

Cholesterol was now examined in two ranges of 0·125 per cent. to 1 per cent. in saline suspensions of 1 in 50 and 1 in 100. When tested with syphilitic sera, no fixation of complement was demonstrated.

Next, a mixture of cholesterol and cardiolipin was tested. The range of cholesterol was again from 0·125 per cent. to 1 per cent., and two sets of mixtures were made with constant cardiolipin contents of 0·15 per cent., and 0·05 per cent. The mixtures were suspended in saline at a strength of 1 in 200. On testing with syphilitic sera, no fixation of complement was obtained with any of these mixtures. It should be noted, however, that in order to carry out these titrations, the syphilitic serum employed must be diluted with normal serum *and not with saline*. If this is not done, the anticomplementary property of the cholesterol-cardiolipin mixture interferes with the experiment (Price, 1953).

Mixtures of the three reagents of cardiolipin Wassermann antigen were now made in which the cardiolipin and lecithin were kept constant at a strength of 0·05 per cent., and the amount of cholesterol was varied. The make-up is shown in Table XIV (overleaf, p. 82).

Quantitative tests with 24 sera were performed using the six antigens at their optimum titres. The results are shown in Table XV (overleaf, p. 82).

It can be seen that when using the comparatively wide range of cholesterol of from 0·3 per cent. to 1 per cent. in the antigen mixtures, the results that were obtained were identical. It was not until antigen 6, containing 0·1 per cent. cholesterol, was employed that there was any marked falling off in the sensitivity of the reaction. This wide zone of the sensitizing effect of cholesterol in cardiolipin

TABLE XIV
KEY TO ANTIGENS USED TO DEMONSTRATE THE COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNTS OF CHOLESTEROL IN CARDIOLIPIN WASSERMANN REACTION ANTIGENS

Antigens	Percent. (and Ratio) of Contents			Optimum Titre	Anticomplementary Titre in Absence of Normal Serum
	Cholesterol	Cardiolipin	Lecithin		
1	1·0 (20)	0·05 (1)	0·05 (1)	1/640	1/40
2	0·8 (16)	0·05 (1)	0·05 (1)	1/480	1/10
3	0·6 (12)	0·05 (1)	0·05 (1)	1/480	1/10
4	0·5 (10)	0·05 (1)	0·05 (1)	1/480	Nil
5	0·3 (6)	0·05 (1)	0·05 (1)	1/240	Nil
6	0·1 (2)	0·05 (1)	0·05 (1)	1/80	Nil

antigens is similar to that which the author has obtained when using the same reagent with the ox-heart alcoholic extracts normally employed for the Wassermann reaction.

The conclusions to be drawn from this section of the work are as follows :

(1) Cholesterol alone does not demonstrate reagin. Mixtures of cholesterol and cardiolipin, and of cholesterol and lecithin, when used as antigens fail to fix complement in the presence of reagin.

(2) The presence of cholesterol in cardiolipin antigens results in a marked increase of sensitivity. As a rough guide, the complement-fixation titres and the P.P.R. titres of the same sera can be compared (see Tables VII, X, XI, XIII, and XV, pp. 78, 79, 80, 81 and 82).

(3) When the strengths of cardiolipin and lecithin are kept constant at 0·05 per cent., and the cholesterol present in the antigens is varied from 0·3 per cent. to 1 per cent., no appreciable difference in the sensitivities of the antigens was demonstrated. If the strength of the cholesterol is 0·1 per cent., there is a marked falling off in the sensitivity of such antigens.

Thus the formulae giving the most sensitive antigens would appear to be within the zone cholesterol 20–6, cardiolipin 1, lecithin 1 (see Tables XIV and XV).

TABLE XV
COMPLEMENT-FIXING EFFECT OF INCREASING AMOUNTS OF CHOLESTEROL IN CARDIOLIPIN WASSERMANN REACTION ANTIGENS

Serum No.	Quantitative P.P.R. Results	Cardiolipin Antigens with Serum Titre Recordings					
		1	2	3	4	5	6
1	1	32	32	32	32	32	8
2	1	32	32	32	32	32–	8–
3	0	32	32	32	32	32	8
4	0	16–	16–	16–	16–	16–	Negative
5	0	32	32	32	32	32	4
6	1	32	32	32	32	32	4
7	0	8–	8–	8–	8–	8–	Negative
8	2	32–	32–	32–	32–	16	Negative
9	2	64–	64–	64–	64–	32	16–
10	1	32	32	32	32	32	Negative
11	4	32	32	32	32	32	Negative
12	2	64	64	64	64	64	16
13	4	64	64	64	64	64	16–
14	1	32	32	32	32	32	4–
15	1	64	64	64	64	64	4–
16	4	64	64	64	64	64–	8–
17	4	32–	32–	32–	32–	32–	4–
18	8	64	64	64	64	64	16
19	2	32–	32–	32–	32–	32–	4–
20	2	32–	32–	32–	32–	32–	4–
21	8	32–	32–	32–	32–	32–	8–
22	8	64	64	64	64	64	16
23	8	64	64	64	64	64	32–
24	8	64	64	64	64	64	32–

Summary

(1) It has been shown that none of the three constituents of cardiolipin Wassermann antigen alone, or mixtures of cardiolipin and cholesterol, or lecithin and cholesterol, when employed as antigens have any ability to fix complement in the presence of syphilitic sera.

(2) (*a*) That mixtures of cardiolipin and lecithin have limited complement-fixing properties when these components are present at suitably adjusted strengths.

(*b*) When cardiolipin and lecithin are present in an antigen at sufficient strengths to demonstrate reagin, an increase in the concentration of cardiolipin raises the sensitivity of the antigen. Under similar conditions an increase in the lecithin content does not *markedly* affect the sensitivity.

(3) Mixtures of the three components of cardiolipin Wassermann antigens fix complement in the presence of reagin over a wide range of strengths of the components :

(*a*) In mixtures of the three components of cardiolipin Wassermann antigen, if the strengths of the lecithin and cholesterol be kept constant and *increasing amounts of cardiolipin* be added, the amount of cardiolipin required for optimum complement-fixation activity can be determined. In the range employed, this was shown to be 0·05 per cent. with a cardiolipin-lecithin-cholesterol ratio of 1-1-10.

(*b*) If the strength of the cardiolipin and cholesterol be kept constant and *increasing amounts of lecithin* be added, the amount required for optimum antigenic activity within the tested range was found to be 0·05 per cent., with a lecithin-cardiolipin-cholesterol ratio of 1-1-10.

(*c*) If the strengths of cardiolipin and lecithin be kept constant at 0·05 per cent. and *increasing amounts of cholesterol* be used, there is not such a sharply defined optimum strength for these antigens. Thus, the antigens are equally sensitive in ratio ranges of cholesterol 20 to 6, cardiolipin 1, lecithin 1. No falling off in the titres of the sera quantitatively tested was shown until the ratio range was somewhere between 6-1-1 and 2-1-1.

(*d*) Using the Whitechapel Wassermann technique, the most sensitive antigen to be employed is one in which the proportion of the reagents is cardiolipin 1, lecithin 1, cholesterol 10–6.

REFERENCE

Price, I. N. Orpwood (1953). *British Journal of Venereal Diseases*, **29**, 15.